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ACTIVE ENDOCANNABINOIDS ARE SECRETED ON EXTRACELLULAR MEMBRANE VESICLES

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Barbara Pauly

1st Editorial Decision 22 May 2014

Thank you for the submission of your manuscript to our editorial office. First of all I would like to apologize for the unusual amount of time it has taken us to get back to you with a decision on your manuscript. We have now received the two enclosed reports on it. In addition, I have discussed your study with an additional advisor of relevant expertise and I am also including an excerpt of his/her comments at the end of this email.

As the detailed reports are pasted below, I would prefer not to repeat them here but you will see that both referees and the advisor have raised substantial concerns about the manuscript and do not recommend its publication in EMBO reports.

The two reviewers raise concerns about the purification and the -in their opinion- insufficient characterization of the vesicles, as well as about the data indicating that the vesicles indeed contain AEAs. Referee 1 also asks whether the vesicles are taken up by the neurons or fuse with the neuronal membrane. Reviewer 3 feels that the study needs to be backed up by experiments in which secretion of the vesicles is blocked and points out that the functional assays have not been performed with the fraction that had been shown to contain the AEAs. Finally, our advisor felt that the physiological relevance of the findings remains insufficiently explored.

Based on these evaluations and the fact that EMBO reports can only invite revisions of papers that receive strong and enthusiastic support from the referees upon initial review, I am afraid I have little choice but to return to you with the decision that EMBO reports cannot publish your manuscript.

I am sorry that I cannot bring better news this time. I hope, however, that the referee comments will be helpful in your continued work in this area and thank you once more for the opportunity to consider your study.

REFEREE REPORTS:

Referee #1:

The authors discuss here the mechanisms by which N-arachidonoylethanolamine (AEA) produced in microglial cells reaches neuronal cells, in order to modulate their activity. The authors propose that extracellular membrane vesicles (EMVs) carry AEA on their surface, and thereby stimulate cannabinoid receptor, with the result that presynaptic transmission is inhibited in GABAergic neurons.
These findings follow previous work of the authors (Antonucci et al., 2012, EMBO J), where they demonstrate that microvesicles of the type used here stimulate glutamatergic synaptic activity. I find the current work interesting, and of substantial importance for the cannabinoid field.
However, one issue needs to be addressed in more detail, before publication. The "extracellular membrane vesicles" are not sufficiently characterized by the authors, and therefore it is difficult to place the findings in a proper perspective.
First, the procedure to isolate these vesicles is not sufficiently described. The authors indicate in the first paragraph of Results that they used "differential centrifugation" for the vesicle isolation. However, they mention that the vesicles "used for electrophysiological and biochemical experiments, were instead obtained at 10.000 g for 30 min" (Methods). Common practice applies the term "differential centrifugation" to centrifugation procedures consisting of more than one step. Therefore it is unclear whether the authors only used this one-step procedure, or a real multiple step differential centrifugation. If the procedure used was indeed only this one step centrifugation, then the results are difficult to trust, since cellular debris may be also present in the pellet.
The authors cite Antonucci et al., 2012 (EMBO J; reference 26) in the section of the Methods that deals with the vesicle isolation procedure. This paper, in turn, cites another work of the authors, Bianco et al., 2009 (EMBO J). This latter paper points the reader to a paper of the Huttner lab (Marzesco et al., 2005, J Cell Sci), but also adds some details in its supplementary section: "The supernatant was then withdrawn and subjected to differential centrifugation at 4 C{degree sign} as follows (all steps at 4{degree sign}C): 5 min at 300g to discard cells and debris (P1 pellet); supernatant, 20 min at 1,200g to obtain P2 vesicle fraction ; supernatant, 30 min at 10,000g to obtain P3 vesicle population; supernatant, 1h at 110,000g to obtain P4 vesicles."(cited from the supplementary data of Bianco et al., 2009, EMBO J). Is this procedure similar to what the authors used here? If not, what exactly was done here?
In addition to this clarification on their methods, the authors need to present a more thorough analysis of the composition of the vesicle fractions. Two questions should be asked:
- Could AEA be found on some other types of organelles, not just EMVs, in this vesicle fraction? And/or in a non-organelle form (lipid droplets, micelle, etc)?
- Is AEA indeed present on the surface of the vesicles, as the authors claim?
The authors may be able to answer such questions by using antibodies against AEA in immunolabeling experiments conducted on the isolated EMVs, in vitro. Several monoclonal lines against AEA have apparently been generated (for example Basta et al., 2004; Journal of Immunological Methods 285: 181-195), although I am not sure whether they can be used in immunolabeling experiments.
Finally, one further point would serve much in the interpretation of the authors' data: it would be important to demonstrate whether the vesicles fuse with the neuronal membranes, or whether they are taken up by the endocytotic system of the neurons. This could be addressed by imaging the particles on the surface of the neurons. Such a system has been developed recently by the laboratory of Mikael Simons, for the study of the effects of myelin particles generated from oligodendrocytes (Bakhti et al., 2013, PNAS). While addressing this type of work in great detail seems beyond the

purposes of the current manuscript, it may not be too difficult to label the EMVs with a lipophilic dye such as PKH26, as performed by the Simons laboratory, and to then image them on the surface of the neurons. If the EMVs are internalized, they will be evident inside the neuronal cell body. If they fuse with the plasma membrane, the lipid dye will diffuse in the entire neuron membrane. If the hypothesis of the authors is right, the EMVs will remain as small discrete spots on the neuronal membranes.

Referee #3:

The manuscript by Gabrielli et al. reports the finding that extracellular vesicles (EV) released by microglial cells contain and functionally transfer the endocannabinoid (eCB) N-arachidonoylethanolamine (AEA) to GABAergic interneurons resulting in a decreased mIPSC frequency. The study addresses a longstanding problem in the eCB field, asking how hydrophobic endocannabinoids (eCBs) cross the hydrophilic extracellular space after their release from postsynaptic neurons or other adjacent cells (such as microglia) to reach their target receptors on the presynaptic terminal. This question is also relevant for the transfer of other lipophilic signaling molecules between cells and thus is of general interest to a broader community. The possibility of vesicle-mediated eCB transport is discussed in the literature and, to my knowledge, this is the first study addressing this important question experimentally. However, the conclusion that AEA indeed is secreted in association with EV is not very well controlled and thus not sufficiently substantiated. Thus, the study is premature requiring more independent lines of experimental evidence to prove its assumption. Therefore, I cannot recommend publication of the manuscript at this stage.

Major comments
1. The standard technique of EV isolation typically includes sequential centrifugation steps and/or filtration to remove cell debris, which were omitted in this study. The authors utilize a crude 100.000 x g sediment of culture supernatants to determine eCBs/AEA. It is essential to include quality controls of the isolated fraction (e.g. biochemical analysis with markers of ectosomes/exosomes as well as markers of organelles that should be absent, nanoparticle tracking analysis). Even with perfect quality control of isolated EV, AEA may be released independently and co-purify with EVs. Thus, it is important to include additional conditions in the analysis, such as interfering with EV secretion (e.g. by inhibition of purinergic signaling or sphingomyelinase). Furthermore, the vesicle fraction could be subjected to specific treatments (pH stress, protease or detergent treatment, repeated freeze thaw cycles) to obtain further insight into the physical nature of the association of AEA with the vesicle fraction. Since the authors mention in the beginning that ATP-treated microglia release ectosomes and exosomes in similar quantities, they even could have discriminated in the analysis between the two to determine which of them actually carries eCB.
2. The most convincing part of the manuscript demonstrates that a 10.000 x g pellet fraction obtained from culture supernatants contains eCB activity by recording mIPSCs from treated hippocampal neurons. However, the vesicle fraction utilized in this functional part of the study is different from that initially tested for presence of AEA. It is not clear how (or if at all) the AEA containing vesicle fraction described in the beginning correlates with the vesicle fraction carrying the activity in the functional assays. Furthermore, it would be important to determine whether the observed decreased mIPSC frequency depends on intact vesicles. Vesicle integrity could be destroyed by mild detergent treatment, osmotic stress or repeated freeze-thaw cycles and the ability to reduce mIPSC frequency compared to intact vesicles.

Advisor's comments:

'... major problem is to understand the relevance of these data. [...] Key results in this study were obtained either from an immortalized ("tumor-like") cell line (microglia N9 cells) or from EMVs isolated by ultracentrifugation from N9 cell supernatants. These EMV were then given to cultures hippocampal neurons. I doubt that these procedures are very physiological and difficult to translate into the in vivo situation. I know that these in vivo experiments would be technical demanding but essentially required to provide evidence of the relevance for their findings'

Correspondence - authors 26 May 2014

Thank you for your letter of May 22 about our manuscript titled "Active endocannabinoids are secreted on extracellular membrane vesicles". Despite your negative decision, we are glad that both Referees considered our work " interesting and of substantial importance for the cannabinoid field" , and described it as " the first study addressing the important question of how lipophilic signaling molecules transfer between cells", and "topic of general interest to a broader community".

We fully agree with the Referees that microvescicle purification is very crucial for data interpretation. As we have been working with extracellular microvesicles for many years, we are fully aware of possible artifacts due to contamination by cellular debris or intracellular organelles and recognize the limits of the methodologies currently available to isolate distinct types of extracellular microvescicles. For these reasons we routinely use an array of methods to isolate and characterize microvesicles. We are very sorry that, due to space limitations, the protocol used to isolate extracellular microvescicles by differential centrifugation was not sufficiently detailed and led the Reviewers to understand that microvesicles were isolated by only one step centrifugation. However, we can easily clarify this misunderstanding and provide the detailed protocols for the isolation of microvesicles using different speeds of centrifugation, their biochemical characterization using markers of ectosomes/exosomes versus different control markers and a description of the quality controls routinely performed to estimate the yield of obtained microvesicles (nanoparticle tracking analysis).

Concerning the Advisor's comments on the functional relevance of our study we would like to point out that N9 cell line-derived microvesicles were used only for first quantification of anandamide content, which was then validated in microvesicles produced by microglial cells in primary cultures. Only microvesicles produced by primary microglia were used throughout the manuscript.

As the presumed insufficient characterization of microvesicles may have heavily impacted on the editorial decision, we respectfully ask you to give us the opportunity to submit a revised version of the manuscript.

Concerning the other comments and constructive criticisms of the two expert Referees, we are willing to perform additional experiments in order to strengthen our manuscript, as we are really convinced that our findings have high impact for the microglia-neuron signalling in brain development and functioning. In response to Referee 1's queries, we already have confocal imaging data showing how microvesicles interact with the surface of neurons and we are willing to perform anandamide immunolabeling experiments on isolated EMVs. According to Referee3's suggestions, we could easily interfere with ectosome secretion by inhibition of purinergic receptors or sphingomyelinase, in order to rule out the possibility that anandamide may be released independently and co-purify with extracellular microvesicles.

We hope that based on these considerations you may allow a new submission of a thoroughly revised version of our manuscript for publication in EMBO Reports

We are looking forward to hearing from you at your earliest convenience.

Correspondence - editor 26 May 2014

Thank you for your email and feedback on our decision not to invite revision of your manuscript. I have now discussed your arguments with my colleagues here at EMBO reports and with our chief editor. We appreciate that you feel you can address the referee concerns about the purification and characterization of the microvesicles.

We would therefore be open to a resubmission of a related manuscript once you have fully addressed their concerns, including the above mentioned issues and additional controls with regard to the vesicle purification. In addition, the other concerns and suggestions of the two reviewers will also have to be fully addressed. These include the suggestion of referee 3 to interfere with vesicle release, to try different treatments of the vesicles to get a better understanding of the association of the endocannabinoids with the exosomes, as well as providing evidence that the vesicles that were used in the functional assays indeed contained endocannabinoids. The concerns of referee 1 would also have to be fully addressed, including additional evidence that the endocannabinoids are indeed present on the vesicle surface and the analysis of whether or not the vesicles fuse with the neurons or are taken up by them.

With regard to the advisor's comment on the physiological relevance of your observations in vivo, we have decided not to insist on these data if all other concerns of the reviewers are adequately addressed.

I would like to stress that such a revised manuscript would be treated as a new submission, also with regard to the novelty of the findings at the time of the submission and would have to be approved by the referees in a second round of review.

Please do let me know if this is an option for you or if you rather seek rapid publication of the study elsewhere.

Correspondence - authors 30 May 2014

Thank you for your positive decision allowing a new submission of a throughly revised manuscript. This is definetely the best option for us.

We will re-submit our manuscript as soon as we have fully addressed the referee's concerns.

1st Revision/Resubmission - authors' response 30 September 2014

Referee 1:

The authors discuss here the mechanisms by which N-arachidonoylethanolamine (AEA) produced in microglial cells reaches neuronal cells, in order to modulate their activity. The authors propose that extracellular membrane vesicles (EMVs) carry AEA on their surface, and thereby stimulate cannabinoid receptor, with the result that presynaptic transmission is inhibited in GABAergic neurons.

These findings follow previous work of the authors (Antonucci et al., 2012, EMBO J), where they

demonstrate that microvesicles of the type used here stimulate glutamatergic synaptic activity. I find the current work interesting, and of substantial importance for the cannabinoid field.

Reply: We thank the Referee for her/his kind appreciation of our work. Hereby we provide a detailed explanation to the changes introduced in the manuscript, in order to provide further information and clarify the issues raised by the Referee.

Comment 1) *However, one issue needs to be addressed in more detail, before publication. The "extracellular membrane vesicles" are not sufficiently characterized by the authors, and therefore it is difficult to place the findings in a proper perspective. First, the procedure to isolate these vesicles is not sufficiently described. The authors indicate in the first paragraph of Results that they used "differential centrifugation" for the vesicle isolation. However, they mention that the vesicles "used for electrophysiological and biochemical experiments, were instead obtained at 10.000 g for 30 min" (Methods). Common practice applies the term "differential centrifugation" to centrifugation procedures consisting of more than one step. Therefore it is unclear whether the authors only used this one-step procedure, or a real multiple step differential centrifugation. If the procedure used was indeed only this one step centrifugation, then the results are difficult to trust, since cellular debris may be also present in the pellet.*
The authors cite Antonucci et al., 2012 (EMBO J; reference 26) in the section of the Methods that deals with the vesicle isolation procedure. This paper, in turn, cites another work of the authors, Bianco et al., 2009 (EMBO J). This latter paper points the reader to a paper of the Huttner lab (Marzesco et al., 2005, J Cell Sci), but also adds some details in its supplementary section: "The supernatant was then withdrawn and subjected to differential centrifugation at 4 C{degree sign} as follows (all steps at 4{degree sign}C): 5 min at 300g to discard cells and debris (P1 pellet); supernatant, 20 min at 1,200g to obtain P2 vesicle fraction ; supernatant, 30 min at 10,000g to obtain P3 vesicle population; supernatant, 1h at 110,000g to obtain P4 vesicles."(cited from the supplementary data of Bianco et al., 2009, EMBO J). ***Is this procedure similar to what the authors used here? If not, what exactly was done here?***
In addition to this clarification on their methods, the authors need to present a more thorough analysis of the composition of the vesicle fractions.

Reply: We fully agree with the Referee that microvescicle purification is very crucial for data interpretation. As we have been working with extracellular microvesicles (EVs) for many years, we are fully aware of possible artifacts due to contamination by cellular debris or intracellular organelles, and recognize the limits of the methodologies currently available to isolate distinct types of EVs. We are very sorry that, due to space limitations, the protocol used to isolate EVs by differential centrifugation was not sufficiently detailed.
The protocol for vesicle isolation has now been detailed in the Methods section (on page 10), and indeed derived from the protocol used by Marzesco et al. 2005 and by Bianco et al. 2009. Briefly, after pre-clearing from cells and debris at 300 g for 10 min (twice), quite large MVs/ectosomes were pelleted from the supernatant by a centrifugation step at 10,000 g for 30 min, while smaller exosomes were subsequently pelletted at higher speed, i.e. 100,000 g for 1h. Alternatively, to collect

a mixed vesicle population for eCB detection, the supernatant pre-cleared from the 300g pellet was centrifugated at 100,000 g for 1h.

Also in response to Referee 3, we have now included in the revised figure 1C western blot analysis of the two vesicle fractions challenged for the ectosome/exosome markers Tsg101, flotillin and alix, as well as for markers of organelles that should be absent, i.e. the mithocondrial marker TOM 20 and the nuclear marker SP1, as quality controls.

Note that in the revised manuscript we use the term extracellular vesicle (EV) instead of extracellular membrane vesicle (EMV) as a generic term for all secreted vesicles and MVs/ectosomes to define vesicles budding from the cell surface to follow the most common nomenclature.

Comment 2) *Two questions should be asked:*

- Could AEA be found on some other types of organelles, not just EMVs, in this vesicle fraction? And/or in a non-organelle form (lipid droplets, micelle, etc)?

Reply: We thank the Referee for her/his comments, which helped us to address this relevant issue.

Firstly, to investigate whether AEA may be released by microglia through non vesicular pathways, such as lipid droplets or micelles which may co-purify with ectosomes, EV fractions collected by ultracentrifugation were analyzed by Western blotting for the specific lipid droplet marker adipophilin (Straub BK et al., 2013 Histopathology). This analysis ruled out possible contamination by lipid accumulations in MV- or exosome- enriched fractions, as shown in the new figure 1C, and described in the text on page 4 and 6.

Secondly, as suggested by Referee 3, we performed new functional assays by exposing neurons to the 10,000g pellet collected under inhibition of MV secretion, i.e. in the presence of the $P2X_7$ receptor antagonist oATP (100 μM), a known blocker of ATP-induced ectosome shedding (Bianco et al., 2005). As shown in the new figure 3F, under this experimental conditions no decrease in mIPSC frequency was evoked by the 10,000g pellet. This finding indicates that MVs do account for the biological activity of the 10.000g pellet. The experiment has been described and discussed on page 6 of the revised text.

Comment 3) *- Is AEA indeed present on the surface of the vesicles, as the authors claim? The authors may be able to answer such questions by using antibodies against AEA in immunolabeling experiments conducted on the isolated EMVs, in vitro. Several monoclonal lines against AEA have apparently been generated (for example Basta et al., 2004; Journal of Immunological Methods 285: 181-195), although I am not sure whether they can be used in immunolabeling experiments.*

Reply: The Referee is right that Basta and colleagues have developed anti-AEA monoclonal antibodies, but as these authors admit *"... the IC50 values in a standard enzyme-linked immunosorbent assay (ELISA) format (ca. 2-3 microM) indicate that improvement in antibody affinities or assay format will be required for an immunoassay to measure endogenous levels. Such work is underway*." Unfortunately, to the best of our knowledge no improvement of these antibodies

has ever been reported, nor any of those anti-AEA mAbs has ever been used in endocannabinoid research. Apart from Basta and coworkers, nobody else has ever reported anti-AEA antibodies that could be used in the immunolabeling experiments suggested by the Referee. Yet, to address her/his point we took advantage of a biotinylated analog of AEA, that we have previously designed for AEA visualization (Fezza F et al., 2008 J lipid Res). Briefly, calcein-labeled MVs, released by microglia preloaded with calcein-AM, were incubated with biotin-AEA (5μM) for 10 min, diluted in KRH and pelleted at 10.000 g before incubation with Cy3-streptavidin for 30 min and further washing in KRH. MVs were then spotted on glass slides, and observed with a Leica SP5 confocal microscope. As shown in the revised figure 4B, incubation with biotin-AEA produced positive labeling of MVs, indicating that AEA can indeed associate to the ectosome surface. No fluorescent signal was detected on MVs exposed to Cy3-streptavidin without previous incubation with biotin-AEA (control). The results and the procedure used to label MVs have been described on page 6 and 12 of the revised text.

Comment 4) *one further point would serve much in the interpretation of the authors' data:* ***it would be important to demonstrate whether the vesicles fuse with the neuronal membranes, or whether they are taken up by the endocytotic system of the neurons****. This could be addressed by imaging the particles on the surface of the neurons. Such a system has been developed recently by the laboratory of Mikael Simons, for the study of the effects of myelin particles generated from oligodendrocytes (Bakhti et al., 2013, PNAS). While addressing this type of work in great detail seems beyond the purposes of the current manuscript, it may not be too difficult to label the EMVs with a lipophilic dye such as PKH26, as performed by the Simons laboratory, and to then image them on the surface of the neurons. If the EMVs are internalized, they will be evident inside the neuronal cell body. If they fuse with the plasma membrane, the lipid dye will diffuse in the entire neuron membrane. If the hypothesis of the authors is right, the EMVs will remain as small discrete spots on the neuronal membranes.*

Reply: Although widely used in our experience PKH dyes can cause artifacts when used to label EVs: PKH dyes form aggregates which can be internalized inside recipient cells, and that are difficult to be distinguished from membrane EVs. In addition, dye aggregates can stain the plasma membrane of recipient cells upon dissolution. Indeed, several fluorescent particles as well as cells with surface PKH dye staining are detectable in negative controls, i.e. recipient cells subjected to the procedure used to label exosomes with PKH dye, in the absence of the ectosomes pellet (see the enclosed figure). Therefore, as an alternative approach to address the Referee's point, we used GFP-labeled MVs. Briefly, neurons were exposed to GFP-labeled ectosomes, produced by glial cells transfected with a plasmid encoding for farnesyl GFP, for 3-h, extensively washed and stained with the plasma membrane-associated protein SNAP-25. Confocal analysis revealed binding of f-GFP-positive MVs to the neural surface. MVs binding occurred along neurites, but also in the somatodendritic compartment of neurons, and was not followed by ectosome internalization, as indicated by the x-y axis projections in revised figure 4A. These findings are described in the revised text on page 6. We are currently investigating by optical manipulation and live confocal

microscopy whether MVs binding to neuronal surface can be followed by fusion with the plasma membrane, by means of MVs labeled with self-quenching concentrations of membrane dye R18, and with the amino-reactive fluorophore AlexaFluor 647 NHS Ester. However, as pointed out by the Referee, addressing what happens after MVs interaction with the surface of neurons goes beyond the purposes of the current manuscript.

Referee 3:

The manuscript by Gabrielli et al. reports the finding that extracellular vesicles (EV) released by microglial cells contain and functionally transfer the endocannabinoid (eCB) N-arachidonoylethanolamine (AEA) to GABAergic interneurons resulting in a decreased mIPSC frequency. The study addresses a longstanding problem in the eCB field, asking how hydrophobic endocannabinoids (eCBs) cross the hydrophilic extracellular space after their release from postsynaptic neurons or other adjacent cells (such as microglia) to reach their target receptors on the presynaptic terminal. This question is also relevant for the transfer of other lipophilic signaling molecules between cells and thus is of general interest to a broader community. The possibility of vesicle-mediated eCB transport is discussed in the literature and, to my knowledge, this is the first study addressing this important question experimentally. However, the conclusion that AEA indeed is secreted in association with EV is not very well controlled and thus not sufficiently substantiated. Thus, the study is premature requiring more independent lines of experimental evidence to prove its assumption. Therefore, I cannot recommend publication of the manuscript at this stage.

Reply: We thank the Referee for considering our work "of general interest to a broader community", and "relevant for the transfer of other lipophilic signaling molecules between cells". Hereby we provide a detailed explanation to the changes introduced in the manuscript, aimed at better substantiating association of AEA to EVs.

Comment 1.1 *The standard technique of EV isolation typically includes sequential centrifugation steps and/or filtration to remove cell debris, which were omitted in this study. The authors utilize a crude 100.000 x g sediment of culture supernatants to determine eCBs/AEA. It is essential to include quality controls of the isolated fraction (e.g. biochemical analysis with markers of ectosomes/exosomes as well as markers of organelles that should be absent, nanoparticle tracking analysis).*

Reply: We appreciated the Referee's expertise on the purification methods and characterization of EVs, and we agree that it is an ongoing issue in the field. We also fully concur with the Referee that EV purification is very crucial for data interpretation. EVs are extensively studied in our lab since many years, and we are fully aware of possible artifacts due to contamination by cellular debris or intracellular organelles; we also recognize the limits of the methodologies currently available to isolate distinct types of EVs. Yet, we are very sorry that, due to space limitations, the protocol used to isolate EVs by differential centrifugation was not sufficiently detailed, and misled the Referee to think that a crude 100.000 x g sediment of culture supernatants was utilized. The detailed protocol for vesicle isolation using different speeds of centrifugation has now been detailed in the Method section (on page 10). It actually derives from the protocol used by Marzesco et al. 2005 and by

Bianco et al. 2009. Briefly, after pre-clearing from cells and debris at 300 g for 10 min (twice), quite large MVs/ectosomes were pelleted from the supernatant by a centrifugation step at 10,000 g for 30 min, while smaller exosomes were subsequently pelletted at higher speed, i.e. 100,000 g for 1h. Alternatively, to collect a mixed population of extracellular vesicles (EVs) for eCB detection, the supernatant pre-cleared from the 300 g pellet was centrifugated at 100,000 g for 1h. Also in response to Referee 1 we have now included in the revised figure 1C western blot analysis of the two vesicle fractions challenged for the ectosome/exosome markers Tsg101, flotillin and alix, as well as for markers of organelles that should be absent, i.e. the mitochondrial marker TOM 20 and the nuclear marker SP1. In addition, we have included in figure 1A the size profiles of MV- and exosome-enriched fractions, evaluated by Nanosight technology, the method we routinely use to estimate the EV yield.

Note that in the revised manuscript we use the term extracellular vesicle (EV) instead of extracellular membrane vesicle (EMV) as a generic term for all secreted vesicles and MVs/ectosomes to define vesicles budding from the cell surface to follow the most common nomenclature.

Comment 1.2. *Even with perfect quality control of isolated EV, AEA may be released independently and co-purify with EVs. Thus, it is important to include additional conditions in the analysis, such as interfering with EV secretion (e.g. by inhibition of purinergic signaling or sphingomyelinase).*

Reply: We thank the Referee for her/his suggestion that helped us to address this relevant issue.

Firstly, to investigate whether AEA may be released by microglia through non vesicular pathways, such as lipid droplets or micelles which may co-purify with MVs, EV fractions collected by ultracentrifugation were analyzed by Western blotting for the specific lipid droplet marker adipophilin (Straub BK et al., 2013 Histopathology). This analysis ruled out possible contamination by lipid accumulations in MV- /exosome- enriched fractions, as shown in the new figure 1C, and described in the text on page 4 and 6.

Secondly, following the Referee' suggestion, we performed new functional assays by exposing neurons to the 10,000 g pellet collected under inhibition of ectosome secretion, i.e. in the presence of the $P2X_7$ receptor antagonist oATP (100 μM), a known blocker of ATP-induced MV shedding (Bianco et al., 2005). As shown in the new figure 3F, under these experimental conditions no decrease in mIPSC frequency was evoked by the 10,000 g pellet. This finding indicates that MVs do account for the biological activity of the 10.000 g pellet. The new experiment has been described and discussed on page 6 of the revised text. Additionally, we could not block ectosome shedding by using imipramine, an inhibitor of acid sphingomyelinase, as the drug likely remains associated to the vesicles surface after washing the pellet with KRH, thus causing altered hippocampal neurotransmission, as previously described by Mendez and colleagues (Mandez P et al., J. Neurosci 2012).

Comment 1.3. *Furthermore, the vesicle fraction could be subjected to specific treatments (pH stress, protease or detergent treatment, repeated freeze thaw cycles) to obtain further insight into the physical nature of the association of AEA with the vesicle fraction.*

Reply: Following the Referee's suggestion, to get insight into AEA-MV interaction and interrogate whether modulation of GABAergic transmission requires intact MVs, we have now analyzed mIPSC frequency from neurons exposed to MVs subjected to repeated freeze thaw cycles. This treatment, which actually breaks MVs without preventing stimulation of excitatory transmission (Antonucci et al., 2012), almost completely abolished the decrease in mIPSC frequency evoked by MVs (see enclosed figure 2). However MVs destroyed by ipo-osmotic stress retained the capability to decrease mIPSC frequency, suggesting that MV integrity is not required for proper presentation of AEA to presynaptic CB_1 (Fig. 4C) and that repeated freeze-thaw cycles likely affect eCB stability. We could not treat MVs with detergents or proteases because residues of detergent/proteases after one wash with PBS were toxic for neurons, and repeated washing and centrifuging may affect vesicle, as well as eCBs, integrity and yield.

Data obtained with MVs broken by iposmotic stress as been included in new figure 4C and in the revised text, on page 6-7.

Comment 1.4. *Since the authors mention in the beginning that ATP-treated microglia release ectosomes and exosomes in similar quantities, they even could have discriminated in the analysis between the two to determine which of them actually carries eCB.*

Reply: To address the Referee's point and determine which type of EVs actually carries AEA, we have now quantified eCBs in MV- and exosome- enriched fractions. This analysis revealed higher AEA levels in ectosomes relative to exosomes. These new data have been described on page 4 of the revised manuscript.

Comment 2. *The most convincing part of the manuscript demonstrates that a 10.000 x g pellet fraction obtained from culture supernatants contains eCB activity by recording mIPSCs from treated hippocampal neurons. However, the vesicle fraction utilized in this functional part of the study is different from that initially tested for presence of AEA. It is not clear how (or if at all) the AEA containing vesicle fraction described in the beginning correlates with the vesicle fraction carrying the activity in the functional assays. Furthermore, it would be important to determine whether the observed decreased mIPSC frequency depends on intact vesicles. Vesicle integrity could be destroyed by mild detergent treatment, osmotic stress or repeated freeze-thaw cycles and the ability to reduce mIPSC frequency compared to intact vesicles.*

Reply: We now clarified on page 4 that all functional studies were performed using MVs/ectosomes, the fraction which contain higher AEA levels (page 4, see also reply to point 1.4 above), and that modulation of mIPSC frequency does not require MV integrity (revised figure 4C), as described in our reply to point 1.3 above.

Figure 1 neurons exposed for 3h to PKH-labelled MVs and corresponding control

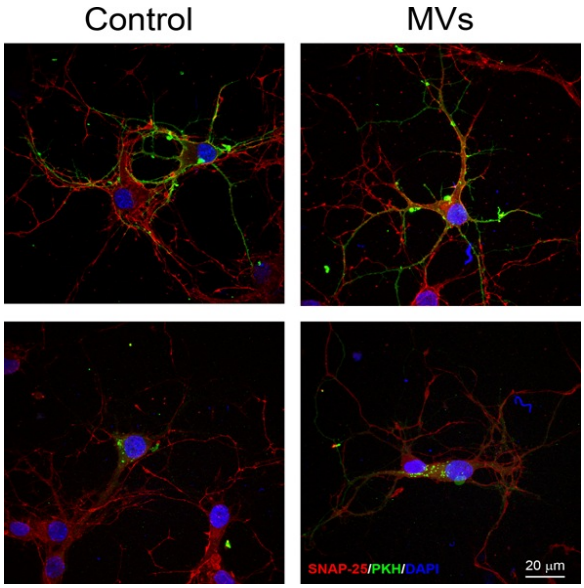
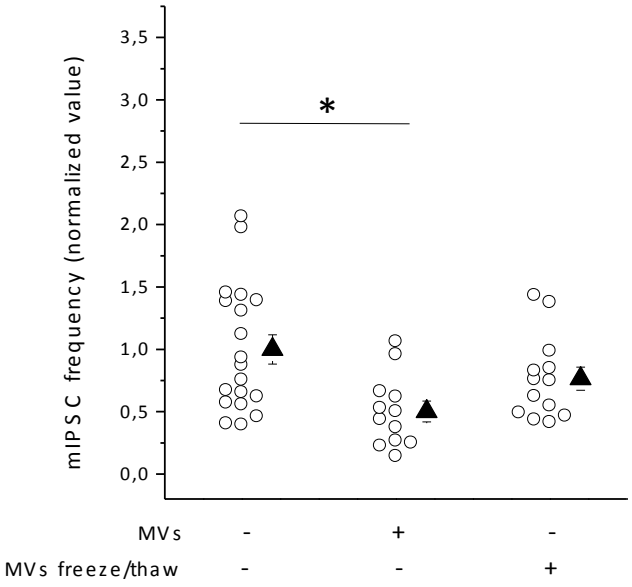


Figure 2 freeze-thaw cycles abolish modulation of mIPSC frequency evoked by MVs

(Kruskal-Wallis ANOVA, P=0.010, Dunn's test for comparison among groups, P < 0.05)



2nd Editorial Decision 22 October 2014

Thank you very much for the submission of your revised manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have now received the reports of the two reviewers who also assessed the first version of it.

Both referees appreciate the additional data that have been added in response to their original reports and in principle support publication of your manuscript. Nevertheless, referee 3 still feels that some issues need to be addressed before this study can be published here. Most of them are rather minor clarifications and additions of controls. However, this reviewer also suggests showing that supernatants from cells that were deprived of microvesicles/exosomes contain lower concentrations of AEAs than control cells. Upon further discussions, referee 1 agrees that these data would strengthen the conclusions of your study and should therefore be added. However, the second point raised by referee 3, namely measuring the concentration of AEAs in the EVs from microglia in which vesicle release has been blocked, is probably not feasible, as interference with vesicle release might damage the cells which would lead to non-specific leakage; we would therefore not insist on you addressing this point.

I would like to ask you to address the remaining concerns as detailed in the attached report, except for the last one mentioned above, and submit a final version of your study.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

The authors have optimally addressed all of my comments, and have revised the manuscript accordingly. I am now happy to suggest publication of the manuscript

Referee #3:

Endocannabinoids (eCB) are paracrine signalling molecules of hydrophobic/lipophilic nature modulating synaptic transmission between neurons. The mode of intercellular eCB trafficking is unresolved and a matter of debate. The manuscript by Gabrielli et al. is a resubmission of a previous manuscript of this group reporting secretion and functional transfer of the eCB arachidonoylethanolamine (AEA) from microglia to neurons via extracellular vesicles (EV). The study demonstrates enrichment of AEA in a crude fraction of mixed EV and provides in vitro proof of principle that EV carry AEA activity to GABAergic hippocampal neurons resulting in decreased inhibition of postsynaptic neurons. The revised manuscript contains additional data with regard to the EV characteristics and demonstrates that inhibition of EV release from microglia results in loss of the functional activity associated with the isolated EV-fraction. Though the manuscript has been improved, the line of argument is still not entirely convincing.

Major points
1. AEA was determined on microvesicles (10.000 g pellets) and exosomes (100.000 g pellets). However, Fig. 1D only depicts crude EV, but should also include the AEA values obtained with MV and exosomes (enrichment factor versus total cells?). These values are only given in the text and it is not clear whether MV and exosomes were derived from N9 cells or primary microglia for AEA determination. It would also be interesting to determine the AEA in the respective supernatants deprived of MV and exosomes to follow AEA enrichment in the MV and exosome fractions. Furthermore, AEA should also be determined in EV fractions derived from microglia treated with oATP inhibiting MV release. This would further support the data presented in Fig. 3D demonstrating restored mIPSC frequency if MV were collected from oATP treated cells.
2. In general, it is not clear whether experiments were performed with MV derived from primary microglia or N9 cells (e.g. NTA Fig. 1A, Fig. 3, Fig. 4). This information is essential and has to be

included in the manuscript at least in the figure legends. The source/fraction of EVs characterized with regard to EV markers and AEA content should match the EV population carrying the activity in the functional experiments.
3. Fig. 4 depicts new data that have been added to demonstrate binding of MV to neurons (4 A) and binding of AEA to the surface of MV. These data are only informative if presented together with negative controls demonstrating specificity of the observed phenomena (MV derived from other cells or artificial vesicles that do not bind to neurons or do not interact with AEA). It would make more sense to determine whether MV binding is observed preferentially in the presynaptic compartment.

Minor points
1. Introduction: the reader should be introduced to the general concept of EC signalling in modulating synaptic transmission (modulation of presynaptic events, retrograde signal).
2. Fig.1A ideally should include NTA measurement of KRH alone to determine the background level of particles present in the buffer (filtration probably does not deplete all particles and particles reform after filtration). Were all samples recorded with the same instrument settings (might be further detailed in the methods section)?
3. Fig. 1C: I'm not sure whether the lipid droplet marker adipophilin is really relevant here. To my opinion it would be more convincing to look at other proteins forming complexes with lipids such as albumin or lipocalins (as mentioned by the authors in the discussion p8). An ER/Golgi marker might be included. Please indicate MW of bands detected.
4. The novelty of information carried by Fig. 2 is limited (might be integrated in Fig. 3 or presented as supplementary material). The use of V-Glut and GAD as markers should be explained to the non-specialist reader.
5. Please explain the basis of mIPSC normalization.
6. The results largely refer to MVs while in the discussion the term ectosomes is prevalent. This may be confusing to the reader.
7. Discussion p8: "we also demonstrate that.....MVs carrying AEA .....inhibit presynaptic release". This is an interpretation of the data and should be expressed accordingly. The data shown in the manuscript focus on post-synaptic events.

2nd Revision - authors' response 28 November 2014

Point-to-point

Major points

1. AEA was determined on microvesicles (10.000 g pellets) and exosomes (100.000 g pellets). However, Fig. 1D only depicts crude EV, but should also include the AEA values obtained with MV and exosomes (enrichment factor versus total cells?). These values are only given in the text and it is not clear whether MV and exosomes were derived from N9 cells or primary microglia for AEA determination. It would also be interesting to determine the AEA in the respective supernatants deprived of MV and exosomes to follow AEA enrichment in the MV and exosome fractions. Furthermore, AEA should also be determined in EV fractions derived from microglia treated with oATP inhibiting MV release. This would further support the data presented in Fig. 3D demonstrating restored mIPSC frequency if MV were collected from oATP treated cells.

   As suggested by the Referee we have now shown in new Fig. 1E AEA content in MVs and exosomes and corresponding values detected in donor primary microglia. In addition, we made clear that these measurements have been carried out on MVs and exosomes produced by primary cells.

   To address the referee query, we attempted to measure AEA in the supernatant deprived of MVs (15 ml sup after 10.000g centrifugation) and deprived of both MVs and exosomes (15 ml sup after 100.000g centrifugation). AEA concentration in these

supernatants remained below the limit of quantification (LOQ) value (LOQ = 0.29 pmol/ml). Due to this technical limitation (eCBs cannot be concentrated) we could not monitor AEA enrichment in MVs and exosomes versus microglia supernatants. We hope that the referee can accept this point. However, it can be speculated that very low AEA concentration in the supernatants deprived of MVs and exosomes are an indirect proof that these vesicles are responsible for AEA trafficking in microglia cells.

2. In general, it is not clear whether experiments were performed with MV derived from primary microglia or N9 cells (e.g. NTA Fig. 1A, Fig. 3, Fig. 4). This information is essential and has to be included in the manuscript at least in the figure legends. The source/fraction of EVs characterized with regard to EV markers and AEA content should match the EV population carrying the activity in the functional experiments.
We have now specified in legends of Fig.1A, and new Fig. 2 and 3 that the experiments were performed in primary microglia. In addition we have made clearer at the end of the first paragraph of the Results that all functional experiments and biochemical/microscope analysis were performed using MVs shed from the microglial surface, i.e. the extracellular vesicle population characterized by higher AEA content.

3. Fig. 4 depicts new data that have been added to demonstrate binding of MV to neurons (4 A) and binding of AEA to the surface of MV. These data are only informative if presented together with negative controls demonstrating specificity of the observed phenomena (MV derived from other cells or artificial vesicles that do not bind to neurons or do not interact with AEA). It would make more sense to determine whether MV binding is observed preferentially in the presynaptic compartment.
It is difficult to identify a good control for microglia-derived MVs, given also MVs derived from non related cultures, i.e. fibroblasts, contain several surface molecules, which may interact with membrane receptors on target neurons (Mulcahy LA JEV 2014). However, specificity of microglial MV adhesion to neurons is indicated by our unpublished videomicroscopy data, obtained using optical manipulation to deliver MVs to neurons, which show that MV adhesion is inhibited by about 50% upon MV treatment with annexin-V. Annexin-V is a high affinity ligand of phosphatidylserine (PS), which is externalized on MV surface and interacts with PS receptors on target neurons, thereby controlling the biological activity of MVs (Antonucci et al., 2012). Addressing how MV interact with neuronal surface goes beyond the purpose of the present work. Therefore we ask not to include these data into the manuscript.
Concerning binding of AEA to the surface of MVs, the experiment was performed to verify whether AEA has affinity for MV membrane and may be exposed on the MV surface in order to activate CB_1 receptors on target neurons. We did not aim at demonstrating that AEA selectively interacts with the surface of MVs released by microglia. Possible interaction of AEA with MVs/exosomes derived from other cell types, especially neurons, may even increase the relevance of our finding, suggesting that EVs may also control retrograde eCB signaling.

Minor points

1. Introduction: the reader should be introduced to the general concept of EC signalling in modulating synaptic transmission (modulation of presynaptic events, retrograde signal).

We thank the Referee for this suggestion. We have now clarified in the introduction that eCBs retrogradely inhibit neurotransmitter release through activation of presynaptic type-1 cannabinoid receptors (CB_1) (page 2 revised introduction).

2. Fig.1A ideally should include NTA measurement of KRH alone to determine the background level of particles present in the buffer (filtration probably does not deplete all particles and particles reform after filtration). Were all samples recorded with the same instrument settings (might be further detailed in the methods section)?
   NTA has been further detailed in (supplementary methods page 2).

3. Fig. 1C: I'm not sure whether the lipid droplet marker adipophilin is really relevant here. To my opinion it would be more convincing to look at other proteins forming complexes with lipids such as albumin or lipocalins (as mentioned by the authors in the discussion p8). An ER/Golgi In previous studies the ability of intracellular albumin to bind AEA has been documented by immobilizing biotin-AEA to fish out all binding proteins; then, identity of bound proteins was disclosed by proteomic analysis. On the other hand, the direct binding of AEA to albumin is very difficult to assess, and remains far from our reach. As far as lipocalin is concerned, this protein has been supposed to bind AEA in solution, but it remains to be demonstrated if and how it does so. Again, it remains out of our reach to run such a direct AEA-protein binding experiment. We hope that the Referee can appreciate the technical difficulty of the requested experiments, that need the development of novel tools (labeled proteins), not yet commercially available.
   Following the Referee's request, we have now included in revised figure 1C western blotting analysis of MVs and exosomes for the golgi marker GS28.

3. The novelty of information carried by Fig. 2 is limited (might be integrated in Fig. 3 or presented as supplementary material). The use of V-Glut and GAD as markers should be explained to the non-specialist reader.
   As suggested by the Referee, Fig.2 is now presented as supplementary Fig.1. In the figure legend we have now explained that the vesicular glutamate transporter V-glut is a marker of excitatory neurons while the glutamic acid decarboxylase GAD is a neuronal marker expressed in inhibitory neurons.

4. Please explain the basis of mIPSC normalization.
   The frequency of mIPSCs is quite variable from culture to culture, reflecting variability in maturation of GABAergic neurons *in vitro* and also within the same neuronal preparation, due to uneven distribution of GABAergic synapses. Normalization of mIPSC frequency to control in each preparation allows to better reveal changes of mIPSC frequency evoked by MVs.

5. The results largely refer to MVs while in the discussion the term ectosomes is prevalent. This may be confusing to the reader.
   To avoid confusion we now always refer to MVs in the Discussion

   Discussion p8: "we also demonstrate that.....MVs carrying AEA .....inhibit presynaptic release". This is an interpretation of the data and should be expressed accordingly. The data shown in the manuscript focus on post-synaptic events.

Reduction of mIPSC frequency but not amplitude is consistent with inhibition of GABA release. However we modified as follows: "We also demonstrate that vesicular eCBs are biologically active, as MVs carrying AEA on their surface activate CB_1 and its downstream signaling in cultured GABAergic neurons, and reduce mIPSC frequency"

3rd Editorial Decision 03 December 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

